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Peripheral NT3 Signaling Is Required for ETS Protein Expression and Central Patterning of Proprioceptive Sensory Afferents

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Summary

To study the role of NT3 in directing axonal projections of proprioceptive dorsal root ganglion (DRG) neurons, *NT3*$^{-/-}$ mice were crossed with mice carrying a targeted deletion of the proapoptotic gene *Bax*. In *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, NT3-dependent neurons survived and expressed the proprioceptive neuronal marker parvalbumin. Initial extension and collateralization of proprioceptive axons into the spinal cord occurred normally, but proprioceptive axons extended only as far as the intermediate spinal cord. This projection defect is similar to the defect in mice lacking the ETS transcription factor ER81 (Arber et al., 2000). Few if any DRG neurons from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice expressed ER81 protein. Expression of a NT3 transgene in muscle restored DRG ER81 expression in *NT3*$^{-/-}$ mice. Finally, addition of NT3 to DRG explant cultures resulted in induction of ER81 protein. Our data indicate that NT3 mediates the formation of proprioceptive afferent-motor neuron connections via regulation of ER81.

Introduction

Neurons of the dorsal root ganglia (DRG) are specialized to convey distinct somatic sensory modalities from the periphery to the central nervous system. Proprioceptive sensory neurons supply skeletal muscle and serve to provide information about muscle length and tension essential for coordinated motor function. Peripherally, group Ia and II proprioceptive neurons terminate on muscle spindles, whereas group Ib afferents innervate Golgi tendon receptors (Zelena, 1994). The central branches of proprioceptors project to the spinal cord and form synaptic connections with interneurons in the intermediate zone of the spinal cord (group Ia, Ib, and II afferents) or directly with motor neurons in the ventral horn of the spinal cord (group Ia and group II afferents; Brown, 1981). The specificity of connections between proprioceptive afferents and motor neurons in the mature spinal cord is well characterized (Eccles et al., 1957; Brown, 1981), but much less is known about the factors regulating proprioceptive axon extension and targeting during development.

Members of the ETS family of transcription factors have been implicated in regulating the formation of synaptic connections between group Ia sensory afferents and motor neurons (Lin et al., 1998; Arber et al., 2000). Two ETS family members, ER81 and PEA3, are expressed by developing proprioceptive sensory neurons as well as by motor neurons in the spinal cord (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002). In the chick embryo, proprioceptive sensory neurons and motor neuron pools projecting to a given muscle exhibit the same pattern of ETS gene expression, and this coordinated expression is regulated by signals from peripheral target tissue (Lin et al., 1998; see also Haase et al., 2002). In mouse, ER81 is initially expressed by most or all proprioceptive neurons in the DRG (Arber et al., 2000). Consistent with this observation, virtually all proprioceptive neurons fail to establish direct monosynaptic connections with motor neurons of *Er81*$^{-/-}$ mice, and terminate instead in the intermediate zone of the spinal cord (Arber et al., 2000). Since proprioceptive neuronal survival is not affected in *Er81*$^{-/-}$ mice, ER81 expression is likely to regulate the expression of molecules necessary for the establishment of the appropriate terminal arborization of group Ia and II afferents within the ventral spinal cord (Arber et al., 2000). Although interactions with the periphery appear to be important in the regulation of ER81 expression (Lin et al., 1998), the identity of the relevant inductive factor(s) responsible for this regulation is unknown.

The two major functional classes of DRG neurons, proprioceptive and cutaneous sensory neurons, are distinguished by their expression of different receptor tyrosine kinases (Trks) that transduce signals provided by different members of the neurotrophin family of polypeptide growth factors (for reviews see Snider, 1994; Bibel and Barde, 2000; Huang and Reichardt, 2001). Neurotrophin signaling via receptor tyrosine kinases underlies, in large part, the target dependence of peripheral neurons during critical developmental periods. Proprioceptive DRG neurons, which comprise ~20% of the adult DRG neuronal population, express TrkC and require neurotrophin-3 (NT3) signaling for their survival during development. Elimination of TrkC signaling in mice results in a 20%–35% loss of DRG neurons and the absence of central proprioceptive projections (Klein et al., 1994; Tessarollo et al., 1994; see also Ernfors et al., 1994; Farinas et al., 1994). In contrast, inactivation of *NT3* in mice results in a ~70% reduction in the number of DRG neurons and thus appears to result in an additional loss of nonproprioceptive sensory neurons (Ernfors et al., 1994; Farinas et al., 1994; Tessarollo et al., 1994).

In addition to their role in regulating neuronal survival,

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there is emerging evidence that neurotrophins have regulatory effects on neuronal morphology, notably functions related to axonal growth and arborization in target fields (for reviews see McAllister et al., 1999; Bibel and Barde, 2000; Huang and Reichardt, 2001; Markus et al., 2002). However, the dependence of peripheral neurons on neurotrophin signaling for survival at early developmental stages has limited our understanding of the requirements for neurotrophins in regulating late developmental events in vivo. Our previous work has demonstrated that genetic elimination of the *Bcl2* family member *Bax*, which is required for apoptosis upon neurotrophin withdrawal (Deckwerth et al., 1996), permits TrkA-expressing cutaneous DRG neurons to survive in the absence of neurotrophin signaling in vivo (Patel et al., 2000). These neurons extend central processes through the dorsal roots into the spinal cord, but the growth of the peripheral process in the limb is markedly impaired (Patel et al., 2000). Thus, elimination of *Bax*, in principle, might provide a tool to explore axon growth and targeting of proprioceptive neurons in the absence of *NT3*.

NT3 appears to play a complex role in the regulation of proprioceptive axon extension and targeting. During development, NT3 is expressed by skeletal muscle, by mesenchyme surrounding peripheral projection pathways and by motor neurons in the spinal cord (Schecterson and Bothwell, 1992; Ernfors et al., 1992; Patapoutian et al., 1999). Injection of blocking anti-NT3 antibodies into the limb during the period of naturally occurring cell death results in a decrease in the number of proprioceptive neurons (Oakley et al., 1995), indicating that NT3 derived from peripheral tissues is required for the survival of proprioceptive neurons. Furthermore, developing peripheral sensory axons can be directed to grow toward local sources of NT3 and other neurotrophins (Tucker et al., 2001), raising the possibility that endogenous NT3 might play a role in supporting interstitial axon extension in the developing limb. It also seems plausible that NT3 derived from either peripheral or spinal cord sources might influence central proprioceptive axon projections. However, it remains to be established whether NT3 regulates the collateralization and targeting of central proprioceptive axons to motor neurons, and if so, by what mechanism.

In order to assess the role of NT3 on proprioceptive axon extension and ER81 expression, we crossed mice carrying a targeted deletion of the *Bax* gene with $NT3^{-/-}$ mice. We report here that proprioceptive sensory neurons survive in $Bax^{-/-}/NT3^{-/-}$ mice. However, peripheral proprioceptive axons and their associated muscle spindles are absent at birth in these mice. Furthermore, although the initial collateralization of central proprioceptive axons into the spinal cord proceeds normally in $Bax^{-/-}/NT3^{-/-}$ mice, these axons do not project toward motor neurons and instead terminate in the intermediate spinal cord, a phenotype similar to that observed in $Er81^{-/-}$ mice (Arber et al., 2000). Consistent with this observation, we find that DRG neurons from $Bax^{-/-}/NT3^{-/-}$ mice express markedly reduced levels of ER81 protein, and we show that exogenous NT3 can induce the expression of ER81 in proprioceptive neurons of DRG explant cultures. Furthermore, we provide evidence that a peripheral rather than a central source of NT3 is required for ER81 expression and central targeting of group Ia and II afferents toward motor neurons.

Results

Elimination of Bax Restores DRG Neuronal Number in the Absence of NT3/Trk Signaling

DRG neurons survive in the absence of neurotrophin signaling in vivo if *Bax* is also deleted (Patel et al., 2000). To study the effects of NT3 on the development of proprioceptive sensory neurons, $Bax^{-/-}/NT3^{-/-}$ mice were generated by crossing heterozygotes from each line. At birth, $Bax^{-/-}/NT3^{-/-}$ mice were overtly indistinguishable from their wild-type littermates. However, $Bax^{-/-}/NT3^{-/-}$ mice did not survive beyond the first postnatal week, as is the case with $NT3^{-/-}$ mice (Ernfors et al., 1994).

We assessed peripheral sensory neuron survival in the progeny of *Bax*/*NT3* crosses by counting lumbar level 4 (L4) DRG neurons in semithin sections at P0 (Figure 1A). As expected, there was a ~70% loss of DRG neurons in $Bax^{+/+}/NT3^{-/-}$ mice compared to wild-type. Strikingly, no neuronal loss occurred in the L4 DRG of $Bax^{-/-}/NT3^{-/-}$ mice. Rather, we found a ~50% increase in the total number of DRG neurons in the $Bax^{-/-}/NT3^{-/-}$ mice (12,932 ± 758), comparable to that found in $Bax^{-/-}/NT3^{+/+}$ mice (14,160 ± 1013). These findings indicate that the elimination of BAX restores neuronal number in the DRG in the absence of NT3. The supranormal number of neurons in both $Bax^{-/-}/NT3^{+/+}$ and $Bax^{-/-}/NT3^{-/-}$ mice is presumably due to the absence of naturally occurring neuron death in the *Bax* null mutants (White et al., 1998; Patel et al., 2000).

To assess whether proprioceptive neurons survived in the absence of NT3 signaling, we examined DRG sections from P0 mice for expression of the calcium binding protein Parvalbumin (Figures 1B–1D). Parvalbumin is an established marker of NT3-dependent proprioceptive neurons (Copray et al., 1994; Ernfors et al., 1994; Honda, 1995). We found Parvalbumin immunoreactivity in large-diameter DRG neurons of wild-type mice (Figure 1B), and consistent with the loss of proprioceptive neurons in $Bax^{+/+}/NT3^{-/-}$ mice, the number of Parvalbumin-expressing neurons in the DRG was dramatically reduced (Figure 1C). In contrast, we found numerous Parvalbumin-expressing neurons in $Bax^{-/-}/NT3^{-/-}$ mice, demonstrating that proprioceptive neurons had survived NT3 deprivation in the absence of *Bax*. However, the cross-sectional area of Parvalbumin$^+$ DRG neuronal somata from $Bax^{-/-}/NT3^{-/-}$ mice was reduced by ~70% as compared to wild-type mice at P0, presumably due to the sustained loss of NT3 trophic support (Figure 1D). Further evidence that proprioceptive neurons survived until P0 in $Bax^{-/-}/NT3^{-/-}$ mice is provided by analysis of their axonal projections into the spinal cord (see below).

A Peripheral Defect in Proprioceptive Axon Projections in $Bax^{-/-}/NT3^{-/-}$ Mice

To determine in $Bax^{-/-}/NT3^{-/-}$ mice whether the peripheral axons of proprioceptive neurons innervate muscles in the absence of NT3 signaling, we searched for the presence of Parvalbumin$^+$ axons in the soleus nerve at P0 (Figure 2). Parvalbumin$^+$ axons were found to extend through the soleus nerve and penetrated the soleus muscle in P0 wild-type ($Bax^{+/+}/NT3^{+/+}$) mice (Figure 2B). In contrast, in the $Bax^{-/-}/NT3^{-/-}$ mice (Figure 2C), no Parvalbumin$^+$ axons were detected in the soleus nerve at P0, despite the fact that Parvalbumin$^+$ axons could be detected in the spinal

A DRG Neuron and Dorsal Root Axon Counts at P0

Genotype	*Bax+/+, NT3+/+*	*Bax-/-, NT3+/+*	*Bax+/+, NT3-/-*	*Bax-/-, NT3-/-*
L4 DRG Neuron Counts	8618 ± 483 (100%)	14,160 ± 1013 (164%)	2531 (30%, n=2)	12,932 ± 758 (150%)
L4 Dorsal Root Axons	8800	13,003	2603 (n=2)	14,485

n=5 for DRG neuron counts; n=3 for dorsal root axon counts

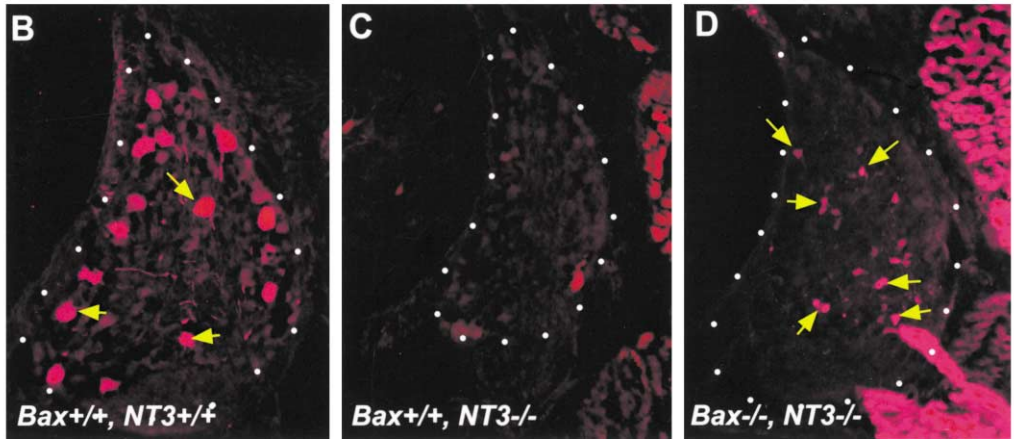

Figure 1. Proprioceptive DRG Neurons Survive in *Bax*$^{-/-}$/*NT3*$^{-/-}$ Mice

(A) L4 DRG neuron and L4 dorsal root axon counts at P0. There is no significant difference in the number of DRG neurons between *Bax*$^{-/-}$/*NT3*$^{+/+}$ (14,160 ± 1013) and *Bax*$^{-/-}$/*NT3*$^{-/-}$ (12,932 ± 758) mice. These values represent a 64% and 50% increase, respectively, over wild-type (8618 ± 483). Note that the number of dorsal root axons corresponds well with the number of DRG neurons for each genotype, demonstrating that the additional DRG neurons in *Bax*$^{-/-}$/*NT3*$^{+/+}$ and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice extend their central axons through the dorsal roots toward the spinal cord.

(B–D) Parvalbumin immunohistochemistry in P0 DRG sections. In wild-type DRG (B), Parvalbumin expression is found in large neurons (arrows), consistent with expression by proprioceptive neurons. As expected, Parvalbumin immunoreactivity is virtually absent in DRG sections from the *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice (C), consistent with the complete loss of proprioceptive neurons in these mice. In contrast, numerous Parvalbumin^{+} neurons can be seen in DRG sections from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (D; arrows). Note, however, that these rescued Parvalbumin^{+} neurons are considerably smaller than Parvalbumin^{+} neurons in wild-type DRG.

cord (see below). Because NT3 could, in principle, regulate the levels of Parvalbumin expression rather than the presence of peripheral axons themselves, we characterized further the extent of peripheral innervation through axon counts in the soleus nerve at P0 (Figure 2A). We detected a 64% decrease in the number of axons in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice when compared with the *Bax*$^{-/-}$/*NT3*$^{+/+}$ mice even though the number of DRG neurons in the two mutants were similar (see Figure 1A). These findings indicate that proprioceptive sensory axons fail to innervate their target muscles in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice.

Muscle spindles are peripheral end organs innervated by proprioceptive neurons, and their development and maintenance are regulated by contacts of group Ia and II afferents with myotubes (Kucera and Walro, 1987, 1988). The morphology and number of muscle spindles in the soleus muscle were identical in *Bax*$^{-/-}$/*NT3*$^{+/+}$ and wild-type mice (Figure 2A). In contrast, we found no muscle spindles in soleus muscles of *Bax*$^{+/+}$/*NT3*$^{-/-}$ or *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice at P0 (Figure 2A). Collectively, the failure to detect Parvalbumin^{+} axons in the soleus nerve, the deficiency in axon number, and the absence of muscle spindles in the soleus muscle demonstrate a developmental failure of proprioceptive neurons to innervate their peripheral muscle targets or an early retraction of these axonal processes.

To distinguish between these two possibilities, we assessed the development of peripheral proprioceptive projections in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos at E15 and E17. E15 represents the earliest stage at which muscle spindle formation in the mouse embryo can be detected, as assessed by the expression of the zinc finger transcription factor Egr3 and the ETS protein Pea3 (Tourtellotte et al., 2001; Hippenmeyer et al., 2002). At E15, we detected Egr3 immunoreactivity in the distal hindlimb muscles of wild-type embryos in approximately 15% of the longitudinal sections examined (n = 3, data not shown). In contrast, in E15 *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we did not detect Egr3 immunoreactivity in distal hindlimb muscles in any of the sections examined (n = 3). At E17, Egr3 is robustly expressed by intrafusal muscle fibers in wild-type embryos, and clusters of Egr3^{+} fibers could be detected in roughly 15% of the sections through both forelimb and hindlimb muscles (arrows in Figures 3A–3C and 3E). In contrast, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos analyzed at E17, no Egr3^{+} fibers were detected in distal forelimb and hindlimb (n = 3; Figures 3D and 3F).

As in P0 mice, no Parvalbumin^{+} axons were detected in hindlimb nerves of *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos at E15 or E17 (data not shown). To assess whether the expression of Parvalbumin may not reveal the full extent of peripheral projections in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we used DiI crystals applied to sciatic nerves as an independent means of tracing peripheral projections. In wild-type embryos, this method readily revealed annulospiral endings characteristic of spindle afferents in soleus and plantaris muscles at

A Soleus Nerve Axon Counts and Soleus Muscle Spindle Counts

Genotype	*Bax+/+, NT3+/+*	*Bax-/-, NT3+/+*	*Bax+/+, NT3-/-*	*Bax-/-, NT3-/-*
Soleus Nerve Axon Counts	78 ± 1	126 ± 23	37 ± 0.6	70 ± 4
Soleus Muscle Spindle Counts	11.2	11.5 (n=2)	0	0

n ≥ 3 for axon and spindle counts

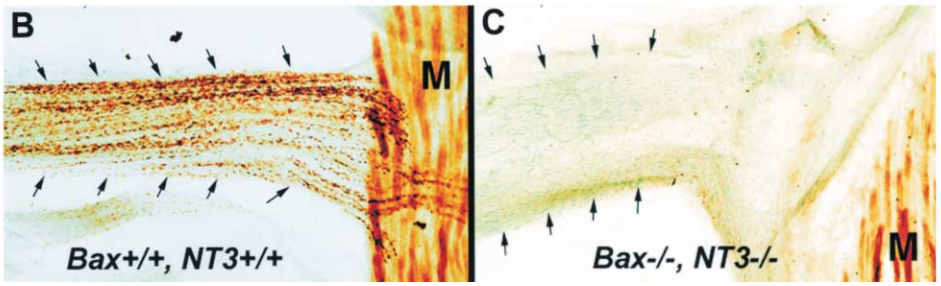

Figure 2. Peripheral Proprioceptive Axons and Muscle Spindles Are Absent at P0 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ Mice

(A) Axon counts in the nerve to the soleus muscle at P0 reveal that the number of axons is reduced in *Bax*$^{-/-}$/*NT3*$^{-/-}$ (70 ± 4) mice compared to *Bax*$^{-/-}$/*NT3*$^{+/+}$ (126 ± 23), even though the number of DRG neurons is equivalent in the two mutants. Furthermore, muscle spindle counts reveal the absence of soleus muscle spindles in *Bax*$^{+/+}$/*NT3*$^{-/-}$ and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice.

(B and C) Parvalbumin^{+} axons can be seen in the nerve to the soleus muscle (outlined by arrows) from wild-type P0 mice. These axons extend through the nerve and penetrate the muscle (M) in the wild-type controls. However, in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, Parvalbumin immunoreactivity is absent in the nerve to the soleus.

E17 (Figure 3G, arrows). Interestingly, some axons with similar morphology were labeled in soleus and plantaris muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, analyzed at the same age (Figures 3H and 3I). These axons were invariably in close proximity to the main nerve trunk and did not extend to lateral aspects of the muscle. Since no Parvalbumin or Egr3 expression could be detected in these preparations, our results do not resolve definitively whether these axons correspond to proprioceptive afferents. Nevertheless, these findings reveal an embryonic defect in the development of peripheral projections of proprioceptive afferents and a corresponding defect in the initiation of muscle spindle differentiation by these proprioceptive afferents.

Proprioceptive Axons Fail to Extend into the Ventral Horn in *Bax*$^{-/-}$/*NT3*$^{-/-}$ Mice

The growth of the central and peripheral axon branches of TrkA-expressing DRG neurons is differentially regulated by NGF signaling (Patel et al., 2000). In order to assess whether proprioceptive neurons extend their axons centrally into the spinal cord in the absence of NT3 signaling, we counted dorsal root axons at P0 by sampling electron micrographs of lumbar dorsal root sections (Figure 1A). Across all genotypes, there was a tight correspondence between DRG neuronal number and dorsal root axon counts, indicating that surviving neurons in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos do extend their central processes into the dorsal roots, toward the spinal cord (Figure 1A).

To characterize further the central projections of proprioceptive afferents, we traced the axons into the spinal cord at E15, by DiI labeling (Figure 4). By E15, proprioceptive afferents have started to invade the ventral horn at all levels of the spinal cord in wild-type mice (Figure 4A). In *Bax*$^{+/+}$/*NT3*$^{-/-}$ embryos, DiI-labeled axons projected only into the dorsal laminae of the spinal cord, consistent with the early death of proprioceptive neurons observed in the absence of NT3 (Figure 4B). In contrast, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, proprioceptive axons entered the spinal cord and followed a trajectory similar to that in wild-type controls (n = 4; Figure 4C, arrows). However, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, proprioceptive axons stopped in the intermediate zone of the spinal cord and failed to project toward motor neurons in the ventral horn (Figure 4, yellow asterisks). Parvalbumin immunostaining in *Bax*$^{-/-}$/*NT3*$^{+/+}$ revealed the presence of proprioceptive afferents in the ventral horn of the spinal cord, indicating that the projection defect observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is due to the absence of NT3 rather than the absence of BAX (data not shown). In order to quantify DiI-labeled proprioceptive afferents, we measured the fluorescence intensity of DiI-labeled afferents in *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Bax*$^{+/+}$/*NT3*$^{+/+}$ mice at E15. The mean fluorescence intensity measurements of DiI-labeled afferents in the intermediate zone of *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice were comparable to the mean density measurements of DiI-labeled afferents in the ventral horn of *Bax*$^{+/+}$/*NT3*$^{+/+}$ mice with a ratio of 1.02 (*Bax*$^{-/-}$/*NT3*$^{-/-}$ versus *Bax*$^{+/+}$/*NT3*$^{+/+}$), indicating that the overall number of proprioceptive collaterals was roughly comparable in the two mutants.

To examine whether axonal projections into the ventral spinal cord were simply delayed developmentally, we traced central projections with DiI at E17 (Figures 4D and 4E) and P0 (Figures 4F and 4G). No afferent projections were detected in the ventral horn at either of these ages in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos (E17, n = 3; P0, n = 2). Parvalbumin staining at P0 verified that these axons were indeed from proprioceptive afferents (n = 3, data not shown).

Proprioceptive DRG Neurons Show Reduced ER81 Expression in the Absence of NT3 Signaling

A defect in the central projections of proprioceptive afferents similar to that in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice occurs in mice lacking the ETS transcription factor *Er81* (Arber et al., 2000). The failure of proprioceptive axons to project

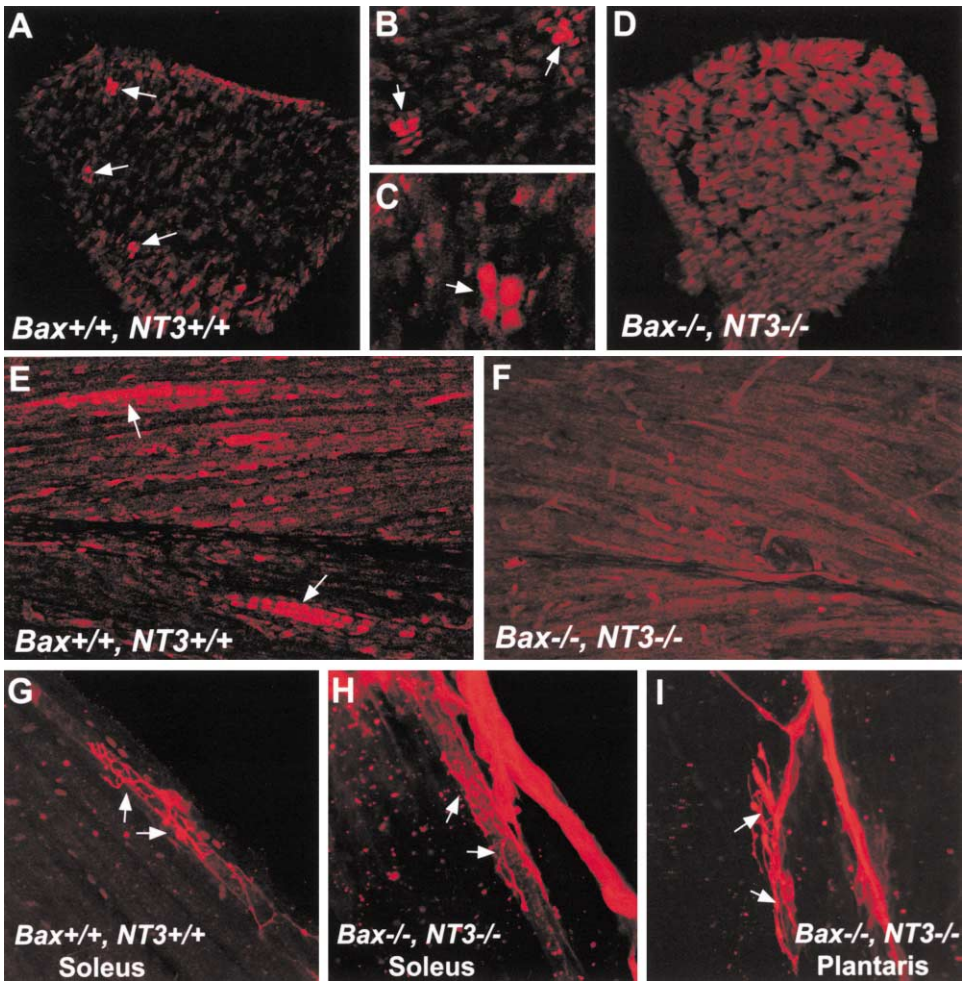

Figure 3. Muscle Spindles Do Not Develop in the Absence of NT3 Signaling in *Bax*$^{-/-}$/*NT3*$^{-/-}$ Mice

(A–F) Egr3 immunoreactivity in distal hindlimb and forelimb skeletal muscle. In cross-sections through wild-type soleus muscle (A–C) and longitudinal sections through the distal forelimb (E), Egr3 immunoreactivity is present in intrafusal muscle fibers at E17. Egr3 immunoreactivity is absent in hindlimb (soleus in D) and forelimb (F) muscles in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice.

(G–I) DiI-labeled peripheral axons in distal hindlimb muscles at E17. The endings of proprioceptive afferents exhibit a characteristic annulospiral structure in the wild-type soleus muscle (G; arrow). A few spiral-like axonal endings are found in the soleus and plantaris muscles from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (H and I, arrows). In contrast to controls, such endings in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice are invariably in close proximity to the major nerve trunk.

into the ventral horn in both *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Er81*$^{-/-}$ mutants raised the possibility that the central projection defect observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is mediated through regulation of ER81 expression. The subset of DRG neurons that express ER81 coexpress TrkC and Parvalbumin and correspond to proprioceptive afferents (Arber et al., 2000). We examined whether proprioceptive DRG neurons expressed ER81 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Unlike at P0, the cross-sectional area of Parvalbumin^{+} DRG neurons in wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice was equivalent at E15 (781 $\pm$ 37 μm^2 in wild-type and 727 $\pm$ 32 μm^2 in *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos; Figures 5A–5C). The intensity of Parvalbumin staining, however, appeared to be fainter in DRG from the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice than wild-type. At E15, numerous ER81+ neurons were detected in DRGs from wild-type (Figure 5E) and *Bax*$^{-/-}$/*NT3*$^{+/+}$ mice (not shown), but no ER81 protein expression was detected in DRG sections from *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos (n = 4; Figure 5G). ER81 expression was also not detected in DRGs of *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice, but this reflects the loss of all proprioceptive neurons (Figure 5F).

To assess whether there was a similar reduction in *Er81* mRNA in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos, we examined *Er81* mRNA levels in E15 lumbar level DRGs by in situ hybridization (n = 2; Figures 5I–5K). Compared to wild-type, we observed a marked reduction in the number of DRG neurons expressing *Er81* mRNA in DRG from *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos. In addition, the intensity of the *Er81* mRNA labeling in individual neurons was reduced in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ DRG compared to wild-type.

The effect of NT3 on ER81 expression appears to be selective to DRG neurons. Subsets of motor neurons in the ventral horn of the spinal cord also express ER81 (Arber et al., 2000). At E15, *Er81* mRNA and ER81 protein (Figures 5L–5O) were expressed in similar patterns in lumbar regions of the spinal cord in wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos. In addition, to assess whether the regulation of ETS genes by NT3 is restricted to ER81, we assessed the expression of Pea3, a member of the ETS

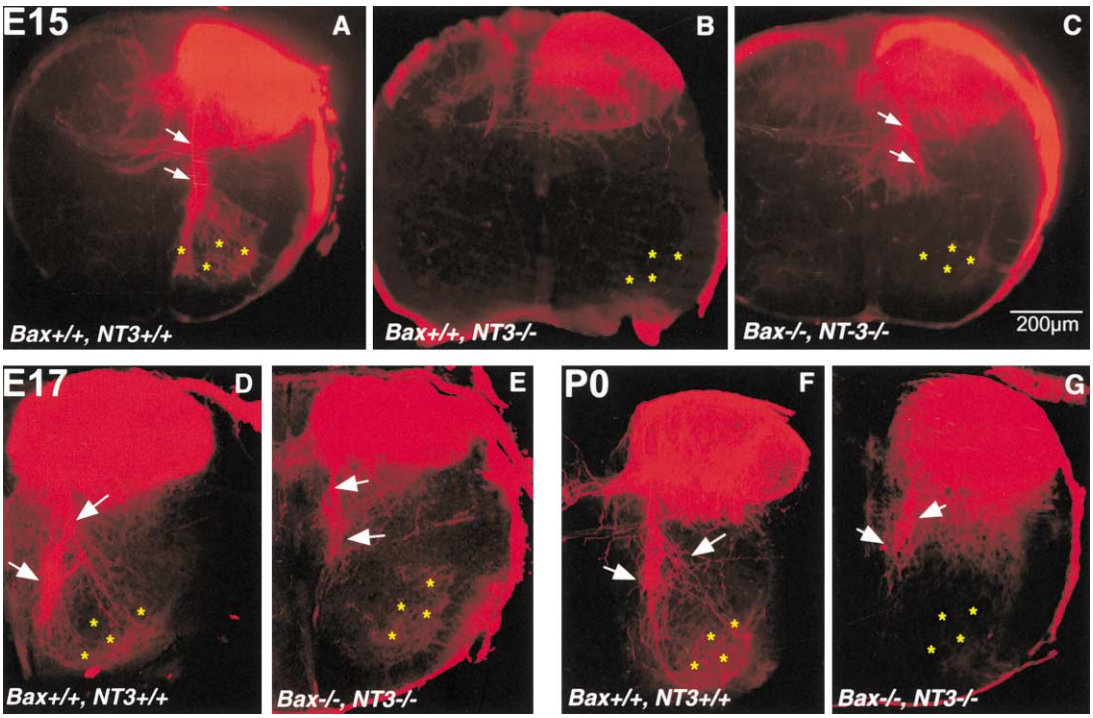

Figure 4. Central Proprioceptive Projections Do Not Reach the Ventral Spinal Cord in *Bax*$^{-/-}$/*NT3*$^{-/-}$ Mice

DiI tracing of the central DRG projections at E15 (A–C), E17 (D and E), and P0 (F and G). In E15 wild-type embryos (A), proprioceptive axons (arrows) penetrate the spinal cord and project ventrally to the motor neurons in the ventral horn (yellow asterisks) of the spinal cord. As expected, the ventrally projecting proprioceptive axons are absent in the spinal cord of E15 *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice (B). In E15 *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (C), the axons (arrows) of the rescued proprioceptive neurons penetrate the spinal cord but extend only as far as the intermediate spinal cord and do not project to the motor neurons in the ventral horn (yellow asterisks). DiI-labeled central projections at E17 (D and E) and P0 (F and G) reveal that the proprioceptive axons of *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice never innervate the motor neurons in the ventral horn of the spinal cord.

gene family closely related to Er81, in DRG neurons of *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos. Pea3 is expressed by both a subset of proprioceptive afferents and a subset of cutaneous DRG neurons (Arber et al., 2000). There was no noticeable difference in patterns or levels of *Pea3* mRNA expression between DRGs from wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ embryos (Figures 5P and 5Q). This result provides evidence for specificity of NT3 in regulating ER81 expression in DRG neurons although it does not absolutely exclude the possibility that Pea3 expression in proprioceptive but not cutaneous sensory neurons is affected.

NT3 Induces ER81 Expression in Proprioceptive DRG Neurons

The absence of ER81 expression in DRG from *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice and the similarity in the central projection phenotype with *Er81*$^{-/-}$ mice raises the possibility that NT3 is responsible for the induction of ER81 in DRG neurons. To test this hypothesis, we cultured wild-type mouse DRG in vitro in the presence or absence of NT3 and assessed ER81 protein expression. Since the onset of ER81 expression in DRG does not occur until E13 in vivo (Arber et al., 2000), DRG explants were cultured from E11.5 and E12.5 (not shown) embryos. We did not detect ER81 immunoreactivity in DRG explants cultured in the absence of NT3 at any time point up to 18 hr even though many DRG neurons expressed the LIM homeodomain protein Isl1. In the presence of NT3, induction of ER81 expression was detected in a subset of Isl1^{+} neurons within 3 hr of culturing the explants, and expression was maintained for up to 18 hr in vitro (Figure 6). In contrast, no expression of ER81 was detected in DRG explants cultured in the presence of NGF for 18 hr (Figure 6).

To rule out the possibility that NT3-dependent DRG neurons die within a few hours of NT3 deprivation in vitro, we cultured E12.5 DRG explants from *Bax*$^{-/-}$ embryos for 18 hr in the presence or absence of NT3. Both NGF- and NT3-dependent neurons from *Bax*$^{-/-}$ DRG neurons survive several days in vitro in the absence of neurotrophin signaling (Lentz et al., 1999). Consistent with the results from wild-type explants, we found numerous ER81^{+} neurons in *Bax*$^{-/-}$ DRG explants cultured in the presence of NT3 for 18 hr in vitro (Figures 7E and 7F). In contrast, few if any ER81^{+} neurons were found in DRG explants cultured in the absence of NT3 (Figures 7B and 7C).

Sources of NT3 Regulating ER81 Expression and the Development of Central Projections of Proprioceptive Neurons

We next addressed the question of whether the source of NT3 responsible for regulating ER81 expression in proprioceptive neurons and the development of ventral projections by group Ia and II afferents is located peripherally or within the spinal cord.

Within the developing spinal cord, motor neurons are known to be a major source of NT3 (see Wright et al.,

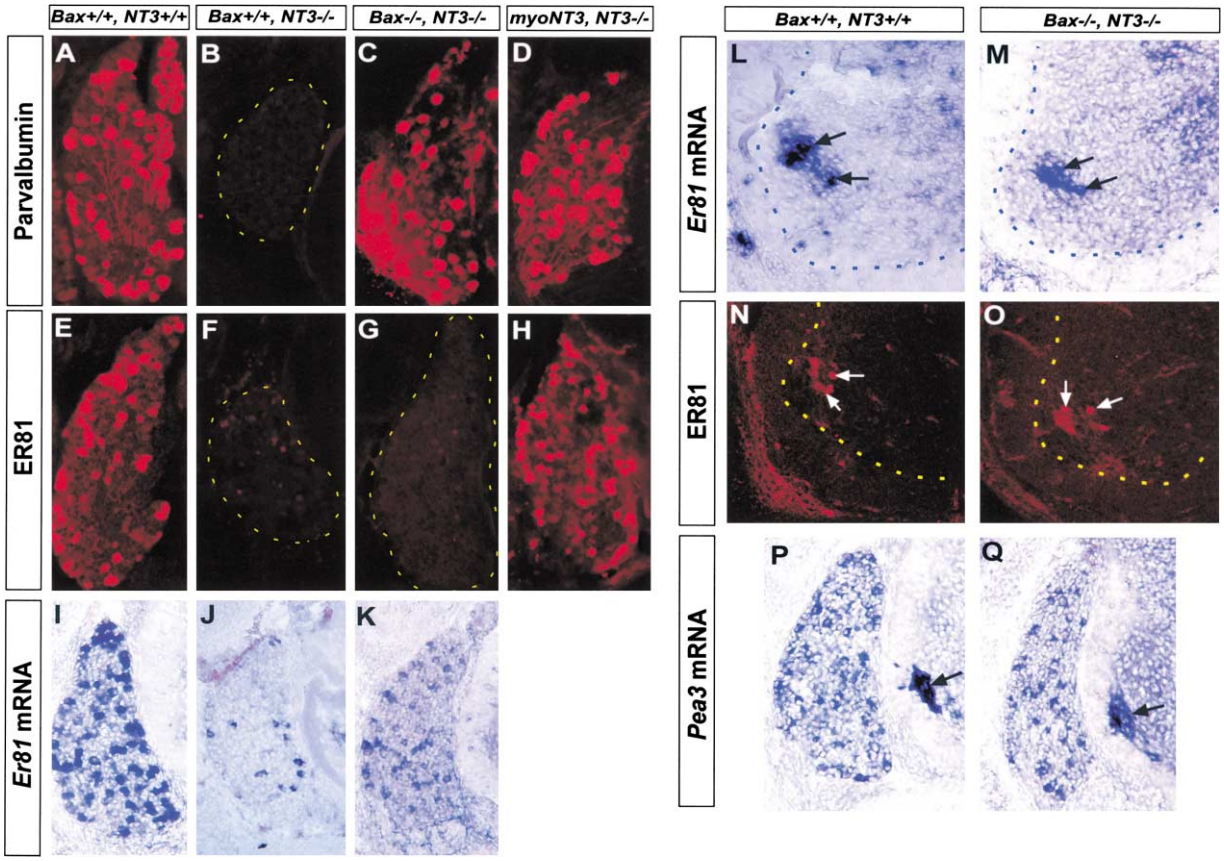

Figure 5. Proprioceptive DRG Neurons Do Not Express ER81 in the Absence of NT3 Signaling

(A–D) Parvalbumin^{+} neurons are found in lumbar DRG sections from E15 wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. As expected, Parvalbumin^{+} neurons are not found in DRG sections from *Bax*$^{+/+}$/*NT3*$^{-/-}$ mice. Parvalbumin-expressing neurons are also rescued in *myoNT3/NT3*$^{-/-}$ mice.

(E–H) Numerous ER81-expressing neurons are present in the wild-type E15 DRG. In contrast, ER81 immunoreactivity is absent in DRG sections from the E15 *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice even though the proprioceptive neurons survive and express Parvalbumin in these mice. ER81 expression is rescued in DRG neurons from *myoNT3/NT3*$^{-/-}$ mice.

(I–K) Consistent with the absence of ER81 immunoreactivity in *Bax*$^{-/-}$/*NT3*$^{-/-}$ DRG neurons, there is also a marked reduction in *Er81* mRNA levels as revealed by in situ hybridization. However, mRNA expression is not abolished in *Bax*$^{-/-}$/*NT3*$^{-/-}$ DRG neurons.

(L–O) In situ hybridization and immunohistochemistry reveal that expression of *Er81* mRNA (L and M) and protein (N and O) in a subset of spinal motor neurons (arrows) is unaffected in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (dots demarcate the boundary of the ventral horn).

(P and Q) In situ hybridization reveals no qualitative difference in pattern or levels *Pea3* mRNA expression between DRGs from E15.5 wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Also note that *Pea3* mRNA is present in motor neurons of both wild-type and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice (arrows).

1997, and references therein). We therefore analyzed a mouse mutant in which motor neurons are ablated genetically by diphtheria toxin expression as soon as they are postmitotic, and thus long before group Ia and II afferents project into the ventral spinal cord (Yang et al., 2001; Pun et al., 2002). In these mice we found normal expression of both ER81 and Parvalbumin in DRG neurons at E17.5 (Figures 8A–8F). Group Ia and II afferents innervated the muscles and were capable of inducing muscle spindles expressing *Pea3* (Figures 8G–8J). In addition, the central projections of proprioceptive afferents in these mice were not impaired in their ability to project into the ventral spinal cord (Figures 8K–8N), arguing against a role for motor neuron-derived NT3 in the induction of ER81 in proprioceptive afferents and the control of the central patterning of group Ia and II afferents.

Second, to assess whether peripheral NT3 is sufficient to restore the expression of ER81 in *NT3*$^{-/-}$ mice, we crossed *NT3*$^{-/-}$ mice with a strain of mice selectively overexpressing NT3 in skeletal muscles under the control of the myogenin promoter (*myoNT3* mice). In *myoNT3/NT3*$^{-/-}$ mice, Parvalbumin^{+} proprioceptive neurons are rescued from apoptotic cell death and these rescued neurons project their axons to the ventral horn of spinal cord (Wright et al., 1997). We examined ER81 expression in lumbar DRGs from E15 *myoNT3/NT3*$^{-/-}$ mice. As reported previously (Wright et al., 1997), Parvalbumin expression was restored by NT3 expression in muscle (Figure 5D). Furthermore, we found that in these animals, numerous DRG neurons express ER81 (Figure 5H).

Finally, in order to define the neuronal cell type in which ER81 exerts its role in controlling the development of central projections of proprioceptive afferents in the ventral spinal cord, we generated a targeted allele of *Er81* in which the first exon coding for the DNA binding ETS domain was flanked by loxP sites (Figure 9A). This mouse strain was crossed to *Isl1*^{Cre} mice to eliminate *Er81* expression exclusively in DRG neurons, motor neurons, and a subpopulation of dorsal interneurons in the spinal cord (Srinivas et

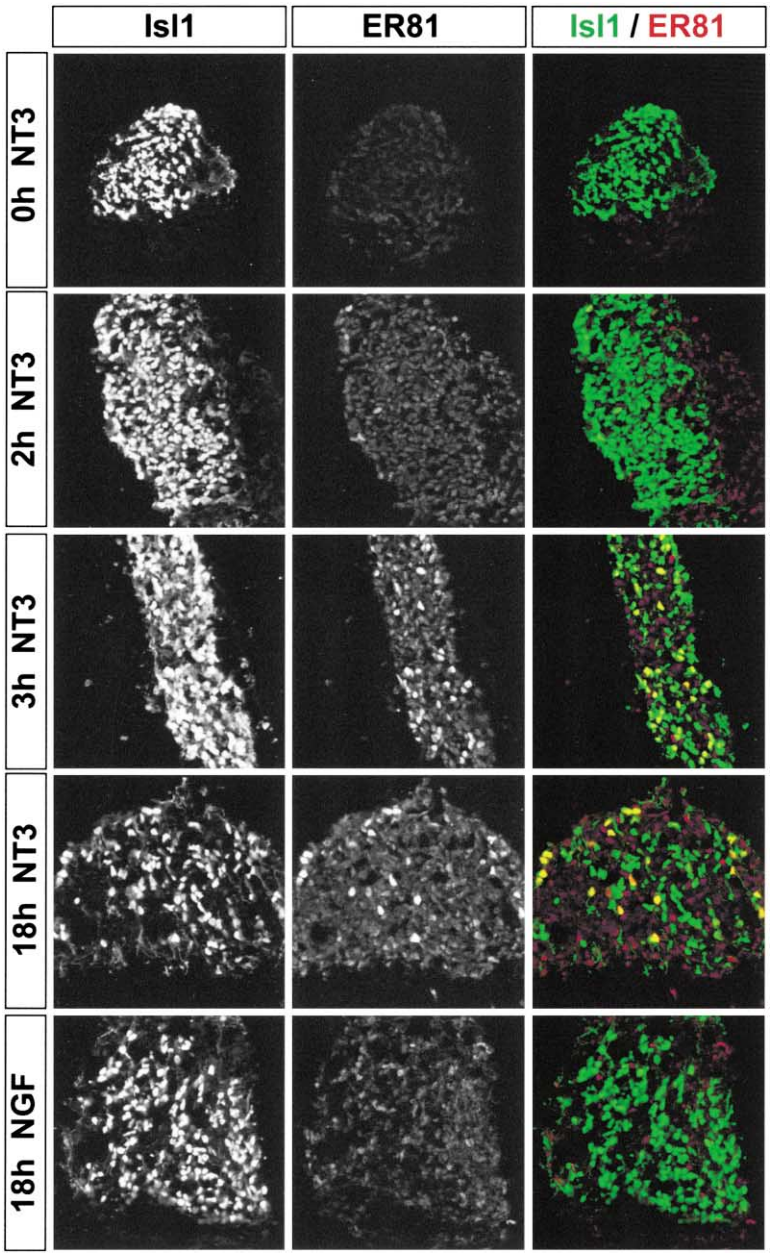

Figure 6. Rapid Induction of ER81 Expression by NT3 in E11.5 DRG Explants

ER81 expression is induced in the presence of NT3 in a subset of Isl1+ DRG neurons within 3 hr of culturing the explants. In the presence of NGF, however, even after 18 hr in vitro, ER81 expression is not induced in DRG neurons.

al., 2001) but not in ER81 expressing interneurons in the intermediate and dorsal spinal cord. In *Isl1^{Cre}/Er81$^{flox/-}$* mice, ER81 protein failed to be expressed in DRG neurons (Figures 9D and 9E), and Parvalbumin^{+} proprioceptive afferents entered the spinal cord appropriately but terminated prematurely in the intermediate zone of the spinal cord (Figure 9G), similar to the findings in constitutive *Er81$^{-/-}$* mice (Arber et al., 2000). These findings thus exclude a role for ER81-expressing interneurons in the intermediate zone of the spinal cord or ER81-expressing muscle spindles in affecting the targeting of proprioceptive afferents to the ventral spinal cord.

Taken together, our findings suggest a model in which NT3 derived from the periphery is required to control expression of ER81 in proprioceptive DRG neurons. This ETS protein in turn controls, in a cell-intrinsic manner, the development of proprioceptive projections to the ventral spinal cord.

Discussion

NT3 is a powerful regulator of DRG neuronal number in vivo and proprioceptive neuron morphology in vitro, but relatively little is known about the effects of NT3 on proprioceptive axon extension and targeting during development in vivo. In this study, we have shown that in *Bax$^{-/-}$/NT3$^{-/-}$* mice, proprioceptive DRG neurons survive through embryonic development. However, the surviving NT3-deprived neurons exhibit both peripheral and central projection defects. The peripheral processes of proprioceptive neurons fail to support muscle spindle differentiation

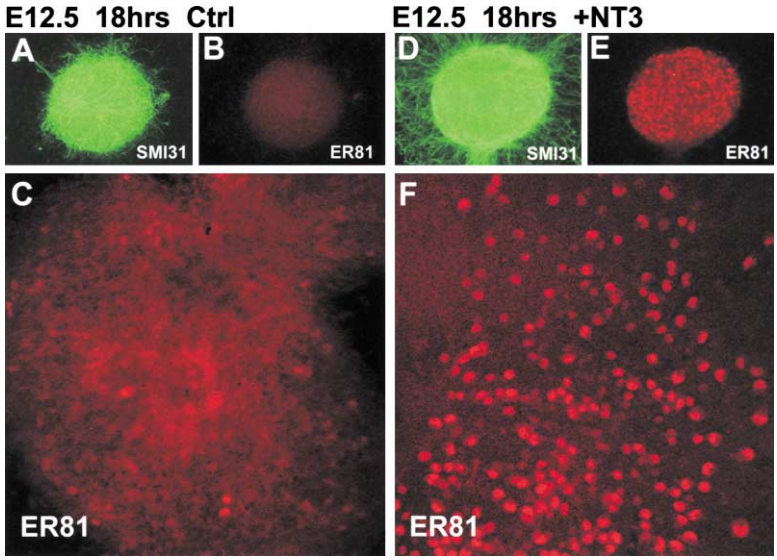

Figure 7. Induction of ER81 Expression in E12.5 *Bax*$^{-/-}$ DRG Explants Is NT3 Dependent

Confocal images of neurofilament (SMI31; [A and D]) and ER81 and staining (B and E) in DRG explants from E12.5 *Bax*$^{-/-}$ mice. ER81^{+} neurons are found after 18 hr in vitro in the presence of NT3 (E and F). Few if any *Bax*$^{-/-}$ DRG neurons exhibit ER81 immunoreactivity in the absence of NT3 (B and C), even after 18 hr. Neurofilament staining demonstrates that *Bax*$^{-/-}$ DRG neurons survive in vitro for 18 hr even in the absence of neurotrophin signaling.

and there is a reduction in the number of axons in the soleus nerve at P0. Central processes extend through the dorsal roots into the dorsal columns and branch and arborize in the intermediate spinal cord but fail to reach the motor neurons in the ventral horn. Furthermore, we find that in the absence of NT3 signaling, proprioceptive DRG neurons do not express the ETS family transcription factor ER81, and conversely NT3 is able to induce ER81 expression in E11 and E12 DRG neurons. Finally, we find that a peripheral source of NT3 is sufficient to induce ER81 expression by DRG neurons in vivo. These findings establish a role for peripheral NT3 in the regulation of proprioceptive afferent projections to motor neuron via the regulation of ER81 expression.

NT3 Regulates DRG Neuron Number via Control of Apoptosis

Our findings demonstrate that elimination of BAX restores DRG neuronal number, as evidenced by a ~50% increase in the number of DRG neurons even in the absence of NT3 signaling. The increase in the number of DRG neurons in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is comparable to that found in *Bax*$^{-/-}$ mice and is presumably due to the elimination of naturally occurring cell death.

There are two important aspects to NT-3 regulation of DRG neuronal number. First, ~20% of DRG neurons, including the entire proprioceptive afferent population, express TrkC from E11 through postnatal life (Mu et al., 1993; Wright and Snider, 1995; White et al., 1996), and 20%–35% of DRG neurons are lost in *TrkC* mutant mice (Klein et al., 1994; Tessarollo et al., 1997). Therefore, NT3/TrkC signaling is thought to regulate survival of proprioceptive neurons in a manner analogous to regulation of the nociceptive population by NGF/TrkA signaling (Crowley et al., 1994; Smeyne et al., 1994). In contrast to findings in *TrkC*$^{-/-}$ mice, *NT3*$^{-/-}$ mice exhibit reductions of 60%–70% in DRG neuron number (Ernfors et al., 1994; Farinas et al., 1994; see also results from this study), raising the question as to the mechanism of action of NT3 on DRG neuron survival at early developmental stages.

The mechanism by which NT3 regulates DRG neuronal number remains unclear but several hypotheses have been put forward to explain the severe neuronal losses observed in the *NT3*$^{-/-}$ mice. Some studies have favored direct regulation of the cell cycle by NT3 (Verdi et al., 1996; Ockel et al., 1996) and therefore imply a control of neuron number through the regulation of precursor cell proliferation. Other studies have suggested that the proliferating precursors in the DRG that express TrkC undergo apoptosis in the absence of NT3, resulting in a depletion of neuronal precursors and in the generation of fewer neurons (ElShamy and Ernfors, 1996). Finally, still other studies of *TrkC*$^{-/-}$ and *NT3*$^{-/-}$ mice have suggested that failure of NT3 to activate TrkB during the proliferation stage of neurogenesis causes DRG precursors to exit the cell cycle prematurely, resulting in a smaller precursor pool (Farinas et al., 1996, 1998). Characterization of Trk protein expression has demonstrated that sensory neuron precursors do not express TrkB or TrkC, suggesting that effects on the proliferating population are indirect (Farinas et al., 1998).

The current findings demonstrate that deletion of *Bax* restores DRG neuron number in the absence of NT3 signaling. Since the *Bax* deletion is thought selectively to affect the ability of DRG neurons or their precursors to enter an apoptotic cell death program and does not influence their capacity to proliferate (Deckwerth et al., 1996; White et al., 1998), our findings suggest that the effects of NT3 on DRG neuronal number are mediated via the inhibition of apoptosis. This conclusion leaves open the question of whether NT3 regulates the survival of precursors and/or postmitotic neurons. It is also possible that the early death of TrkB and TrkC neurons, or their precursors, could decrease the proliferation of neighboring cells. Our results do, however, argue against the idea that the direct regulation of proliferation by NT3 is likely to be a critical determinant of DRG neuronal number.

NT3 Regulates Peripheral Components of the Proprioceptive System

Proprioceptive DRG neurons survive in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, but we find that their peripheral projections and associated muscle spindles are absent at P0. Thus, in the

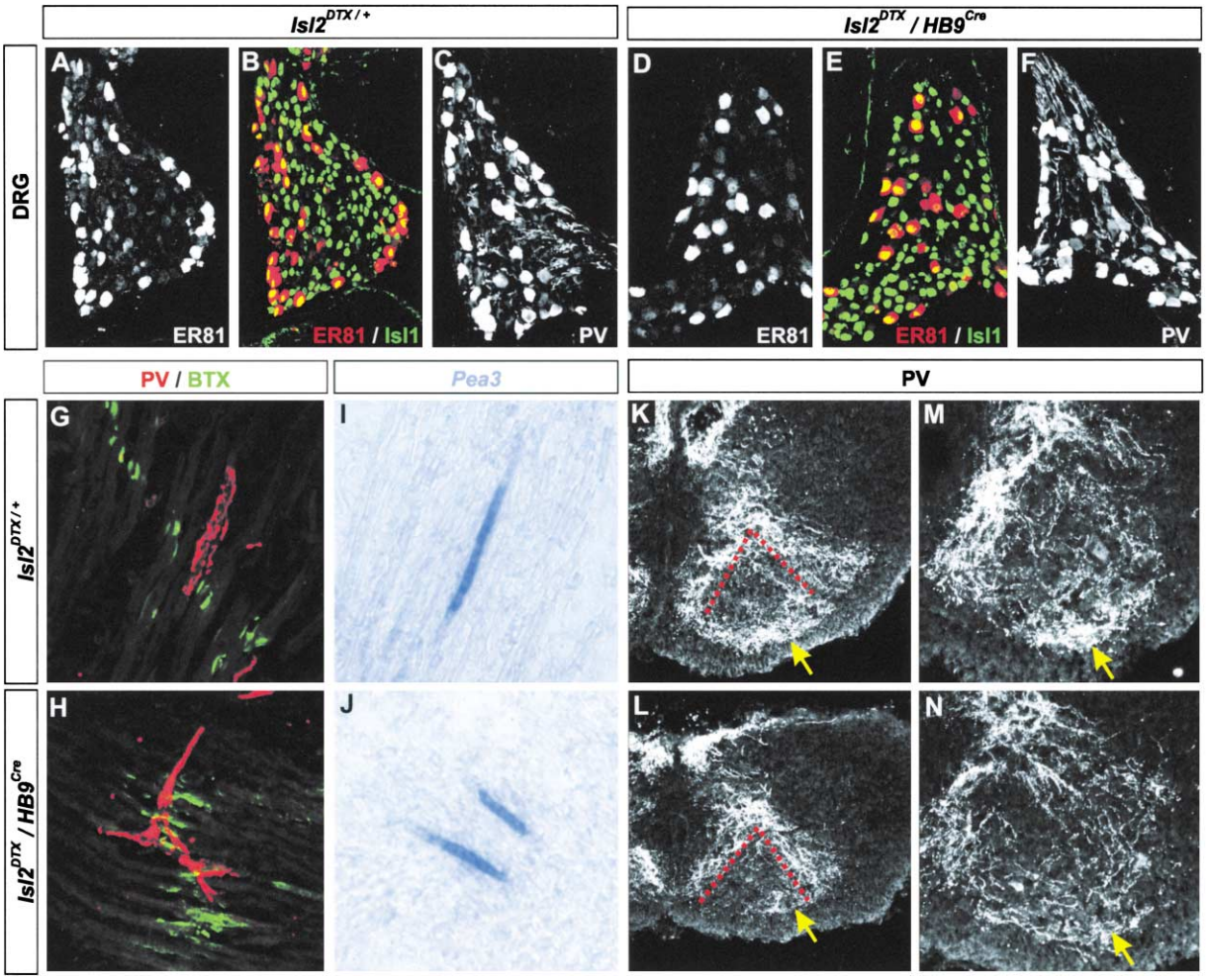

Figure 8. Proprioceptive Afferent Development in the Absence of Motor Neurons

(A–F) ER81 (A and D), ER81 and Isl1 (B and E), and Parvalbumin (C and F) immunocytochemistry on E17.5 brachial DRG from *Isl2*^{DTX} (A–C) and *Isl2*^{DTX}/*Hb9*^{Cre} (D–F) embryos.

(G–J) Analysis of muscle spindle differentiation by Parvalbumin (PV) and α-bungarotoxin (G and H) immunocytochemistry or *Pea3* in situ hybridization (I and J) on forelimb muscles of E17.5 *Isl2*^{DTX} (G and I) and *Isl2*^{DTX}/*Hb9*^{Cre} (H and J) embryos.

(K–N) Analysis of central projections of proprioceptive afferents by Parvalbumin immunocytochemistry on spinal cords of E17.5 *Isl2*^{DTX} (K and M) and *Isl2*^{DTX}/*Hb9*^{Cre} (L and N) embryos. Arrows point to the ventral horn of the spinal cord where group Ia and II afferents normally terminate.

absence of NT3 signaling, there is a failure of peripheral proprioceptive axons either to grow toward their skeletal muscle targets during development, or alternatively a retraction of these processes with subsequent degeneration of the muscle spindles. Analysis of the expression of the zinc finger transcription factor Egr3 (Tourtellotte and Milbrandt, 1998) in muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$ at E15 and E17 revealed no staining for Egr3, arguing that Egr3 expression is never initiated in these mice. While we also never detected Parvalbumin^{+} proprioceptive afferents in muscles of *Bax*$^{-/-}$/*NT3*$^{-/-}$, which may be caused by the low expression level of Parvalbumin by these afferents, an independent tracing experiment revealed some peripheral axons in distal limb muscles with a morphology similar to annulospiral endings of group Ia or II afferents. An intriguing possibility would thus be that proprioceptive afferents are present transiently in muscle nerves of *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, but do not release Neuregulin-1, which is thought to mediate initiation of muscle spindle differentiation (Hippenmeyer et al., 2002).

The lack of appropriate development of peripheral endings of proprioceptive afferents and associated sensory organs observed in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is analogous to our previous observations in *Bax*$^{-/-}$/*NGF*$^{-/-}$ mice in which TrkA+ nociceptive DRG neurons survive but their peripheral cutaneous projections are absent at P0 (Patel et al., 2000). Together, these findings suggest a generality in the principle that neurotrophins are essential for the establishment and/or maintenance of peripheral sensory projections in vivo.

NT3 Regulates Proprioceptive Afferent Projections into the Ventral Spinal Cord via Regulation of ER81 Expression

A striking finding of this study is that proprioceptive group Ia and II afferents fail to extend into the ventral spinal cord in the absence of NT3 signaling. Nevertheless, the initial extension of proprioceptive afferents into the dorsal roots and their initial arborization into the dorsal spinal cord

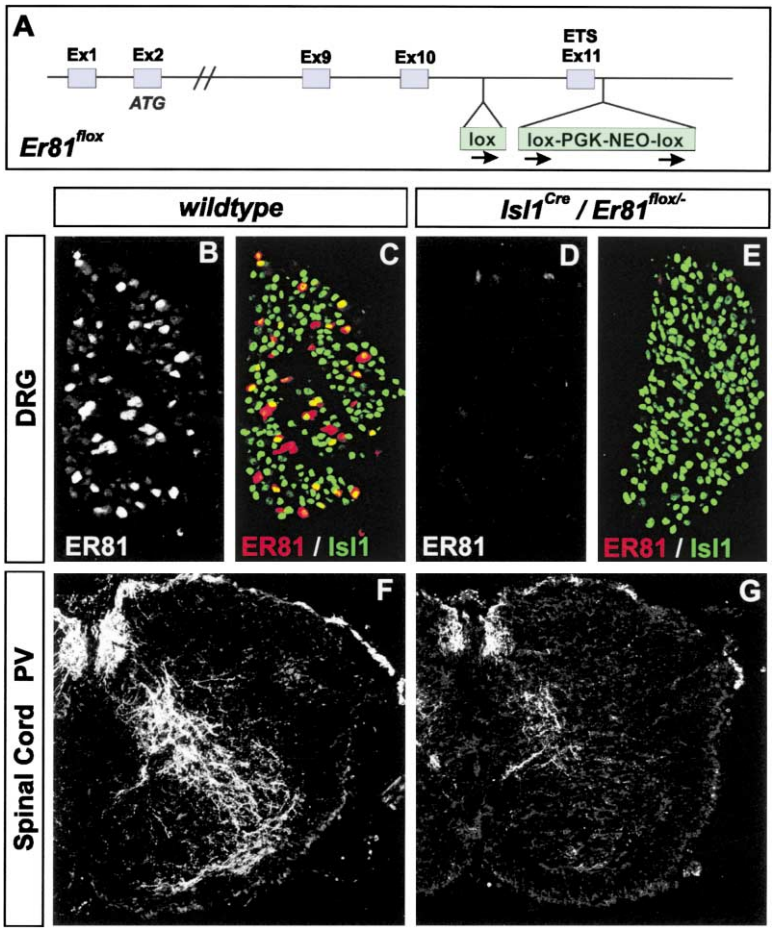

Figure 9. ER81 Acts in Proprioceptive Afferents to Control Central Connectivity

(A) Schematic diagram of targeting strategy for the *Er81*^{flox} allele.

(B–E) ER81 (B and D) and ER81/Isl1 (C and E) immunocytochemistry on E17.5 lumbar DRG from wild-type (B and C) and *Isl1*^{Cre}/*Er81*$^{flox/-}$ (D and E) embryos demonstrates absence of ER81 protein in DRG of *Isl1*^{Cre}/*Er81*$^{flox/-}$.

(F and G) Analysis of central projections of proprioceptive afferents by Parvalbumin immunocytochemistry on E17.5 lumbar spinal cords of wild-type (F) and *Isl1*^{Cre}/*Er81*$^{flox/-}$ (G) embryos.

proceed normally in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mutants. This finding is consistent with our observations in *Bax*$^{-/-}$/*NGF*$^{-/-}$ and *Bax*$^{-/-}$/*TrkA*$^{-/-}$ mice in which the extension of the central nociceptive projections into spinal cord proceeds normally in the absence of NGF/TrkA signaling (Patel et al., 2000). Since neither NT3 nor NGF are expressed in the dorsal roots or the dorsal horn of the spinal cord, the extension of the central sensory axonal processes appears not to require neurotrophin signaling.

Why, in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, do the central group Ia and II projections extend only as far as the intermediate spinal cord and fail to project into the ventral horn? The targeting defect in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice is similar to that seen in mice lacking the ETS family transcription factor ER81. We find that proprioceptive DRG neurons in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ do not express ER81 protein and that ER81 expression can be induced rapidly by application of NT3 in DRG explant cultures. NT3 expression by motor neurons does not appear to be required for the induction of ER81 in DRG. Moreover, peripheral NT3 can restore both the expression of ER81 in DRG as well as the development of central projections of proprioceptive afferents into the ventral spinal cord in *NT3*$^{-/-}$ mice. Since selective elimination of ER81 in DRG and motor neurons recapitulated the *Er81*$^{-/-}$ phenotype, ER81 expression by interneurons in the intermediate zone of the spinal cord and by intrafusal muscle fibers is not crucial to proprioceptive-motor neuron targeting. Together, these findings suggest that peripheral NT3 regulates central proprioceptive afferent projections through its ability to regulate ER81 in proprioceptive afferents.

ER81 is a member of the ETS family of transcription factors, an evolutionary conserved gene family characterized by sequence homology within the DNA binding ETS domain (reviewed in Bartel et al., 2000). It is unclear how ER81 regulates the targeting of proprioceptive afferents to motor neurons in the ventral horn of the spinal cord, but there is growing evidence that ETS family members play a role in regulating late steps in the establishment of sensory-motoneuron connectivity. In *Pea3*$^{-/-}$ mice, the axons of specific pools of motor neurons fail to invade and branch normally within their target muscles and the cell bodies of these motor neurons are mispositioned within the spinal cord (Livet et al., 2002). Limb-derived signals, mediated in part by GDNF, regulate the expression of ETS proteins in motor neurons (Livet et al., 2002; Haase et al., 2002). Thus, an important implication of our findings, together with studies on PEA3, is that ETS genes act cooperatively at a late step of development to establish functional sensory-motor circuitry. In turn, ETS gene expression is regulated by neurotrophic factors released from peripheral target tissues.

It is interesting that NT3 regulates ER81 expression in proprioceptive sensory but not spinal motor neurons even though both of these neuronal classes express the NT3 receptor TrkC during embryonic development (Yan et al.,

1993). This is one of several examples of differences in NT3 actions on these two types of neurons; for example, unlike proprioceptors, motor neurons do not require NT3 for survival. Little is known about differences in Trk intracellular signaling mediators that may be responsible for neuron class-specific effects.

One important question is the identity of downstream targets of ER81 that regulate the projection of proprioceptive axons. Given the specificity of the central proprioceptive targeting defect in *Er81*$^{-/-}$ and *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, ER81 may be involved in regulating the expression of axon guidance and/or cell recognition molecules. Candidates include the type II cadherins. In developing chick DRG, type II cadherin family members are coexpressed with ETS family members ER81 and PEA3 in proprioceptive neurons and spinal motor neurons. A matching of cadherin expression in proprioceptive sensory and motor neurons could therefore provide a basis for the selectivity with which sensory-motoneuron connections are formed (Price et al., 2002). It is possible that NT3 regulation of ER81 expression regulates the expression of type II cadherins in proprioceptive DRG neurons. Indeed, ectopic expression of ER81 results in the deregulation of at least one type II cadherin family member in the chick spinal cord (Price et al., 2002), and *Pea3*$^{-/-}$ mice show an altered profile of type II cadherin expression in motor neurons (Livet et al., 2002).

Finally, ER81 is unlikely to be the only transcription factor involved in controlling the development of central connectivity of group Ia and II afferents. Recently, a Runx family transcription factor Runx3 has been shown to be an essential regulator of proprioceptive DRG neuron development (Levanon et al., 2002; Inoue et al., 2002). *Runx3* mutant mice exhibit severe limb ataxia due to disruption of monosynaptic connectivity between proprioceptive afferents and motor neurons. The central projection defect in the *Runx3*$^{-/-}$ mice is more severe than that reported in *Er81*$^{-/-}$ mice (Arber et al., 2000) and than that reported here in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Specifically, in *Runx3*$^{-/-}$ mice, proprioceptive DRG neurons fail to extend central processes into the intermediate spinal cord. In contrast, in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ and *Er81*$^{-/-}$ mice, proprioceptive afferents extend as far as the intermediate zone but fail to extend into the ventral horn. These findings raise the possibility that *Runx3* and ER81 function coordinately for proper targeting of group Ia and II afferents to the ventral horn.

Roles of Neurotrophic Factors in Axon Targeting

Our study and the prior studies of Haase et al. (2002) and Ma et al. (2002) establish an important role for neurotrophic molecules in regulating axon targeting. Surprisingly, their effect on targeting is not due to the intensively studied chemotropic function of these molecules (see O'Connor and Tessier-Lavigne, 1999; Tucker et al., 2001, and references therein). In the circuit considered here, NT3 is expressed by motor neurons well prior to projection of proprioceptive afferents into the ventral horn (see Wright et al., 1997, and references therein). We show here that proprioceptive afferents project toward the ventral horn even if motor neurons are ablated genetically. In addition, it has been shown that a central source of NT3 is unimportant in the central targeting of proprioceptive axons since central NT3 can be neutralized by anti-NT3 antibodies without affecting the projections of proprioceptive afferents (Oakley et al., 1995). Our evidence demonstrates that NT3 regulates targeting of proprioceptive afferents to motor neurons via a different mechanism—the regulation of ER81 expression and presumed subsequent effects on gene transcription through a peripheral source of NT3. At a later stage, local regulation of proprioceptive axon branching in the vicinity of the motor pools may involve Wnt signals derived from motor neurons (Krylova et al., 2002).

Since there is a peripheral projection defect in *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice, it could also be argued that target-derived factors in addition to NT3 are required to direct proprioceptive afferents to the ventral horn. Two lines of evidence suggest that NT3 has the predominant role. In chick, when proprioceptive axons are deprived of their peripheral targets by limb bud ablation, exogenous application of NT3 is sufficient to direct projection of muscle afferents appropriately within the spinal cord (Oakley et al., 1997). Furthermore, peripherally supplied NT3 is sufficient to direct central projections to motor pools even if the peripheral proprioceptive axon is misrouted in the periphery and therefore exposed to atypical peripheral influences (Oakley and Karpinski, 2002). Interestingly, target-derived NT3 also exerts important influences on proprioceptive afferent-motor neuron connections at later developmental stages and into maturity. Thus, intramuscular injections of NT3 rescue the functional deficit in group Ia and II synaptic transmission observed in *Egr3*$^{-/-}$ mice where proprioceptors lose their peripheral target end organ due to progressive degeneration of muscle spindles after birth (Tourtellotte and Milbrandt, 1998; Chen et al., 2002). Furthermore, administration of NT3 rescues the defect in group Ia and II synaptic transmission that results from separation of proprioceptive afferents from their targets by axotomy in fully mature animals (Mendell et al., 1999). Thus, a single target-derived neurotrophic factor appears to regulate both the development and function of this sensory-motor circuit.

Experimental Procedures

Animals

Bax$^{-/-}$ mice on a 129/Bl6 background (from Dr. Stan Korsemeyer) were crossed with *NT3*$^{+/-}$ mice also on a 129/Bl6 background to generate *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. *Bax*$^{-/-}$ and *NT3*$^{-/-}$ mice maintained on a pure C57Bl/6 genetic background (Jackson Labs) were also crossed to produce *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice. Results from the two genetic backgrounds were indistinguishable. Genotypes were confirmed by tail DNA PCR (Deckwerth et al., 1996; Wright et al., 1997). *Er81*^{flox} mice were generated by the integration of *loxP* sites 5′ and 3′ of Exon11 in the *Er81* locus in a targeting strategy analogous to Arber et al. (2000). *Isl1*^{Cre}, *Hb9*^{Cre}, and *Isl2*^{DTX} mice have been described previously (Srinivas et al., 2001; Yang et al., 2001; Pun et al., 2002). The generation of *myo-NT3* mice was described previously (Wright et al., 1997).

Neuron and Nerve Fiber Counts

Neuron and axon counts in the different mutants were conducted as described previously in detail in Patel et al. (2000).

To measure the cross-sectional area of Parvalbumin^{+} DRG neurons at E15 and P0, images of lumber DRG sections were captured at 20× magnification and analyzed using the NIH image software. A minimum of 15 lumbar DRG sections from each mouse was captured. The outline of Parvalbumin^{+} cell bodies were manually traced and diameter and cross-sectional area measurements were recorded.

Immunohistochemistry

E15, E17, and P0 mice were intracardially perfused with 4% paraformaldehyde (PFA). Tissue was dissected, postfixed in 4% PFA,

washed extensively in PBS, and incubated in 30% sucrose/PBS. DRG, spinal cord, and limb sections were cut on a cryostat at 10 to 20 μm. Sections were incubated in primary antibody overnight. The following antibodies were used: goat anti-Parvalbumin (1:500, Swant, Switzerland), guinea pig anti-Isl1 (Arber et al., 2000), rabbit anti-Parvalbumin (1:5000, Swant, Switzerland), rabbit anti-Egr3 (1:500, Santa Cruz, Santa Cruz, CA), and rabbit anti-ER81 (1:12,500; Arber et al., 2000). Sections were washed in PBS and incubated with the appropriate Cy-2- or Cy-3-conjugated secondary antibodies (Jackson ImmunoReaserch, West Grove, PA), Alexa-488-conjugated secondary antibodies (Molecular Probes), or fluorescently labeled α-bungarotoxin (Molecular Probes) and prepared for visualization. To confirm the absence of Egr3 immunoreactivity in E15 and E17 *Bax*$^{-/-}$/*NT3*$^{-/-}$ muscles, we examined more than 100 hindlimb and forelimb sections at E15 and more than 200 sections at E17 from a total of three animals at each age.

DiI Labeling

To label the central DRG axon projections, DiI crystals (Molecular Probes) were placed directly in the DRG at E15, E17, and P0. The tissue was incubated at 37°C in 4% paraformaldehyde and monitored periodically to assess the extent of labeling. Spinal cords were sectioned at 75 μm on a vibratome for visualization. For labeling of peripheral axon projections, DiI crystals were placed in the sciatic nerve proximal to the tibial and common peroneal branch points of E17 embryos and processed as above.

To quantify DiI labeled afferents, mean intensity measurements in defined regions of the intermediate zone and ventral horn of spinal cord were obtained using the NIH Image analysis software. Photoshop images were first converted to gray scale and inverted. Images were then imported into NIH Image, and mean intensity measurements of DiI-labeled afferents in a given area were recorded. Background corrections were made for each section. Measurements were made in a fixed area at a defined point for all sections examined. The data were expressed as the ratio of mean density measurements in the *Bax*$^{-/-}$/*NT3*$^{-/-}$ mice versus the *Bax*$^{+/+}$/*NT*$^{+/+}$ mice.

In Situ Hybridization

In situ hybridization was performed on fixed tissue sections according to previously described protocols (Wright and Snider, 1995; Patel et al., 2000) using digoxigenin-labeled sense and antisense riboprobes for *Er81* and *Pea3*.

Explant Cultures

For ER81 induction studies, E11.5 and E12.5 wild-type DRG were dissected and whole explants cultured in DMEM/F12 medium supplemented with 10% FCS and 2 mM L-glutamine. Cultures were maintained under conditions of no neurotrophin, 10 ng/ml NT3, or 50 ng/ml NGF up to 18 hr. At different time intervals, explants were washed once for 5 min in PBS, fixed for 20 min on ice in 4% PFA, washed again in PBS, incubated in 30% sucrose/PBS overnight, and embedded the next morning using the Tissue-Tek OCT compound. After freezing, 9-μm-thick sections were cut and antibody staining performed according to standard procedures.

Bax$^{-/-}$ DRG explant cultures were performed as described in Patel et al. (2000).

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References

Arber, S., Ladle, D.R., Lin, J.H., Frank, E., and Jessell, T.M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell *101*, 485–498.

Bartel, F.O., Higuchi, T., and Spyropoulos, D.D. (2000). Mouse models in the study of the Ets family of transcription factors. Oncogene *19*, 6443–6454.

Bibel, M., and Barde, Y.A. (2000). Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. *14*, 2919–2937.

Brown, A.G. (1981). Organization in the Spinal Cord (New York: Springer).

Chen, H.H., Tourtellotte, W.G., and Frank, E. (2002). Muscle spindle-derived neurotrophin 3 regulates synaptic connectivity between muscle sensory and motor neurons. J. Neurosci. *22*, 3512–3519.

Copray, J.C., Mantingh-Otter, I.J., and Brouwer, N. (1994). Expression of calcium-binding proteins in the neurotrophin-3-dependent subpopulation of rat embryonic dorsal root ganglion cells in culture. Brain Res. Dev. Brain Res. *81*, 57–65.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., MacMahon, S.B., Shelton, D.L., Levinson, A.D., et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell *76*, 1001–1011.

Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, E.M., Jr., Snider, W.D., and Korsmeyer, S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron *17*, 401–411.

Eccles, J.C., Eccles, R.M., and Lundberg, A. (1957). The convergence of monosynaptic excitatory afferents onto many different species of alpha motoneurones. J. Physiol. *137*, 22–50.

ElShamy, W.M., and Ernfors, P. (1996). A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. Neuron *16*, 963–972.

Ernfors, P., Merlio, J.P., and Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Eur. J. Neurosci. *4*, 1140–1158.

Ernfors, P., Lee, K.F., Kucera, J., and Jaenisch, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell *77*, 503–512.

Farinas, I., Jones, K.R., Backus, C., Wang, X.Y., and Reichardt, L.F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature *369*, 658–661.

Farinas, I., Yoshida, C.K., Backus, C., and Reichardt, L.F. (1996). Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron *17*, 1065–1078.

Farinas, I., Wilkinson, G.A., Backus, C., Reichardt, L.F., and Patapoutian, A. (1998). Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo. Neuron *21*, 325–334.

Haase, G., Dessaud, E., Garcès, A., de Bovie, B., Birling, M.-C., Filippi, P., Schmalbruch, H., Arber, S., and DeLapeyrière, O. (2002). GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron *35*, 893–905.

Hippenmeyer, S., Shneider, N.A., Birchmeier, C., Burden, S.J., Jessell, T.M., and Arber, S. (2002). A role for neuregulin1 signaling in muscle spindle differentiation. Neuron *36*, 1035–1049.

Honda, C.N. (1995). Differential distribution of calbindin-D28k and parvalbumin in somatic and visceral sensory neurons. Neuroscience *68*, 883–892.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. *24*, 677–736.

Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S.C., et al. (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. *5*, 946–954.

Klein, R., Silos-Santiago, I., Smeyne, R.J., Lira, S.A., Brambilla, R., Bryant, S., Zhang, L., Snider, W.D., and Barbacid, M. (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature *368*, 249–251.

Krylova, O., Herreros, J., Cleverley, K.E., Ehler, E., Henriquez, J.P., Hughes, S.M., and Salinas, P.C. (2002). WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. Neuron *35*, 1043–1056.

Kucera, J., and Walro, J.M. (1987). Postnatal maturation of spindles in deafferented rat soleus muscles. Anat. Embryol. (Berl.) *176*, 449–461.

Kucera, J., and Walro, J.M. (1988). The effect of neonatal deafferentation or deefferentation on the immunocytochemistry of muscle spindles in the rat. Neurosci. Lett. *95*, 88–92.

Lentz, S.I., Knudson, C.M., Korsmeyer, S.J., and Snider, W.D. (1999). Neurotrophins support the development of diverse sensory axon morphologies. J. Neurosci. *19*, 1038–1048.

Levanon, D., Bettoun, D., Harris-Cerruti, C., Woolf, E., Negreanu, V., Eilam, R., Bernstein, Y., Goldenberg, D., Xiao, C., Fliegauf, M., et al. (2002). The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. EMBO J. *21*, 3454–3463.

Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessell, T.M., and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell *95*, 393–407.

Livet, J., Sigrist, M., Stroebel, S., De Paolo, V., Price, S.R., Henderson, C.E., Jessell, T.M., and Arber, S. (2002). ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron *35*, 877–892.

Ma, L., Harada, T., Harada, C., Romero, M., Hebert, J.M., McConnell, S.K., and Parada, L.F. (2002). Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. Neuron *36*, 623–634.

Markus, A., Zhong, J., and Snider, W.D. (2002). Raf and akt mediate distinct aspects of sensory axon growth. Neuron *35*, 65–76.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. *22*, 295–318.

Mendell, L.M., Johnson, R.D., and Munson, J.B. (1999). Neurotrophin modulation of the monosynaptic reflex after peripheral nerve transection. J. Neurosci. *19*, 3162–3170.

Mu, X., Silos-Santiago, I., Carroll, S.L., and Snider, W.D. (1993). Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J. Neurosci. *13*, 4029–4041.

Oakley, R.A., and Karpinski, B.A. (2002). Target-independent specification of proprioceptive sensory neurons. Dev. Biol. *249*, 255–269.

Oakley, R.A., Garner, A.S., Large, T.H., and Frank, E. (1995). Muscle sensory neurons require neurotrophin-3 from peripheral tissues during the period of normal cell death. Development *121*, 1341–1350.

Oakley, R.A., Lefcort, F.B., Clary, D.O., Reichardt, L.F., Prevette, D., Oppenheim, R.W., and Frank, E. (1997). Neurotrophin-3 promotes the differentiation of muscle spindle afferents in the absence of peripheral targets. J. Neurosci. *17*, 4262–4274.

Ockel, M., Lewin, G.R., and Barde, Y.A. (1996). In vivo effects of neurotrophin-3 during sensory neurogenesis. Development *122*, 301–307.

O'Connor, R., and Tessier-Lavigne, M. (1999). Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. Neuron *24*, 165–178.

Patapoutian, A., Backus, C., Kispert, A., and Reichardt, L.F. (1999). Regulation of neurotrophin-3 expression by epithelial-mesenchymal interactions: the role of Wnt factors. Science *283*, 1180–1183.

Patel, T.D., Jackman, A., Rice, F.L., Kucera, J., and Snider, W.D. (2000). Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. Neuron *25*, 345–357.

Pun, S., Sigrist, M., Santos, A.F., Ruegg, M.A., Sanes, J.R., Jessell, T.M., Arber, S., and Caroni, P. (2002). An intrinsic distinction in neuromuscular junction assembly and maintenance in different skeletal muscles. Neuron *34*, 357–370.

Price, S.R., De Marco Garcia, N.V., Ranscht, B., and Jessell, T.M. (2002). Regulation of motor neuron pool sorting by differential expression of type II cadherins. Cell *109*, 205–216.

Schecterson, L.C., and Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron *9*, 449–463.

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature *368*, 246–249.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell *77*, 627–638.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Constantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Tessarollo, L., Vogel, K.S., Palko, M.E., Reid, S.W., and Parada, L.F. (1994). Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. Proc. Natl. Acad. Sci. USA *91*, 11844–11848.

Tessarollo, L., Tsoulfas, P., Donovan, M.J., Palko, M.E., Blair-Flynn, J., Hempstead, B.L., and Parada, L.F. (1997). Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. Proc. Natl. Acad. Sci. USA *94*, 14776–14781.

Tourtellotte, W.G., and Milbrandt, J. (1998). Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. Nat. Genet. *20*, 87–91.

Tourtellotte, W.G., Keller-Peck, C., Milbrandt, J., and Kucera, J. (2001). The transcription factor Egr3 modulates sensory axon-myotube interactions during muscle spindle morphogenesis. Dev. Biol. *232*, 388–399.

Tucker, K.L., Meyer, M., and Barde, Y.A. (2001). Neurotrophins are required for nerve growth during development. Nat. Neurosci. *4*, 29–37.

Verdi, J.M., Groves, A.K., Farinas, I., Jones, K., Marchionni, M.A., Reichardt, L.F., and Anderson, D.J. (1996). A reciprocal cell-cell interaction mediated by NT-3 and neuregulins controls the early survival and development of sympathetic neuroblasts. Neuron *16*, 515–527.

White, F.A., Silos-Santiago, I., Molliver, D.C., Nishimura, M., Phillips, H., Barbacid, M., and Snider, W.D. (1996). Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. J. Neurosci. *16*, 4662–4672.

White, F.A., Keller-Peck, C.R., Knudson, C.M., Korsmeyer, S.J., and Snider, W.D. (1998). Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. J. Neurosci. *18*, 1428–1439.

Wright, D.E., and Snider, W.D. (1995). Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. J. Comp. Neurol. *351*, 329–338.

Wright, D.E., Zhou, L., Kucera, J., and Snider, W.D. (1997). Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. Neuron *19*, 503–517.

Yan, Q., Elliott, J.L., Matheson, C., Sun, J., Zhang, L., Mu, X., Rex, K.L., and Snider, W.D. (1993). Influences of neurotrophins on mammalian motoneurons in vivo. J. Neurobiol. *24*, 1555–1577.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T.M., Birchmeier, C., and Burden, S.J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron *30*, 399–410.

Zelena, J. (1994). Nerves and Mechanoreceptors—The Role of Innervation in the Development and Maintenance of Mammalian Mechanoreceptors (New York: Chapman and Hall).